Abstract

Focal adhesions are structures that secure the cell to the surrounding extracellular matrix. Immunofluorescence staining of vinculin is a common technique that allows for quantification of focal adhesion size and quantity. A protocol for staining focal adhesions in vascular smooth muscle cells (VSMCs) was synthesized through a review of relevant literature and optimized for performance. Process optimization demonstrated that an incubation period in 2% bovine serum albumin prevents non-specific binding, therefore, allowing for the mouse vinculin monoclonal antibody to bind more readily to vinculin and vinculin only in VSMCs. ImageJ software was used to measure an average focal adhesion length of 32.302 μm in standard VSMCs. The optimized protocol for immunofluorescence labeling of vinculin will be used in future applications and by other lab personnel to better inform our understanding of cellular structures that contribute to mechanical integrity.

Introduction

Understanding the mechanical properties of VSMCs reveals information about their differing behavior in health and disease. Recently, research efforts have focused on understanding the behavior of VSMCs following traumatic brain injury (TBI). VSMCs are deformed by 15 % strain and by a strain rate of up to 1000 %/s during TBI, which affects their ability to contract and regulate blood flow in the brain [1]. Thus, more information about VSMC response to high-speed deformation is necessary to better understand the implications of TBI. In prior experiments where VSMCs were stretched to a fixed strain at varying strain rates, stress was lower in VSMCs when they were stretched at high strain rates. These results were unexpected. Cells are commonly modeled as viscoelastic materials. In viscoelastic materials, stress increases as strain rate increases.

This finding suggests that VSMCs do not exhibit viscoelastic behavior [1]. This finding suggests there may be outlying biophysical mechanisms within the cell that affect VSMC post-deformation behavior.

The cell is attached to the extracellular matrix through focal adhesions, structures that maintain the cell's geometry during deformation. VSMCs contract in response to deformation. Since VSMC deformation modeling is performed under conditions where the VSMCs are bound to the extracellular matrix, it is important to understand if focal adhesions play a significant role in the unexpected, non-viscoelastic behavior of VSMCs [2]. Focal adhesions are composed of a complex network of proteins. A major component of focal adhesions is vinculin [3]. Understanding how the rate of deformation affects the size and number of focal adhesions in VSMCs may help explain experimental observations of decrease in contractile stress. In this experiment, I developed and optimized a protocol for measuring the size and number of focal adhesions in VSMCs through fluorescent staining of vinculin.

Methods

Human umbilical artery VSMCs were seeded onto polyacrylamide gel constructs stamped with aspect ratio 1:4 fibronectin islands to facilitate cell adhesion and most closely capture the in vivo structure of VSMCs. The VSMCs were cultured overnight in supplemented Medium-199 (Gibco) and incubated at 37 °C and 5% CO_2. 24 hours before fixing the cells, the cell culture medium was replaced with serum-free Medium-199 to promote a contractile phenotype in the VSMCs [1].

Cells were fixed with 4% paraformaldehyde for 15 minutes, followed by permeabilization with 0.2% Triton X-100 at room temperature for 5 minutes. The following variables were tested

for efficacy in staining vinculin adhesion: 1) dilution of the primary antibody; 2) dilution of the secondary antibody; and 3) method for blocking non-specific binding. The primary antibody used in these experiments was a mouse vinculin monoclonal antibody. VSMCs were stained for 1 hour at room temperature with the following dilutions: 1:100 and 1:500. After staining with the primary antibody, VSMCs were stained with a secondary antibody. The secondary antibody used in these experiments was an Alexa fluor 488 anti-mouse antibody. VSMCs were stained for 1 hour at room temperature at the following dilutions: 1:50, 1:100, 1:200, and 1:250. Dilutions of the primary and secondary antibodies were performed by diluting the antibodies in either PBS or a blocking solution of 2% bovine serum albumin (BSA). Additionally, it was tested whether a 1-hour incubation with 2% BSA before any antibody staining would provide better results. After 3 washes with PBS, the VSMCs were ready for focal adhesion imaging [4]. The cells were imaged at 40x magnification under a FITC filter using a confocal microscope [1]. The number of vinculin-positive focal adhesions for each construct was analyzed using ImageJ software [5].

Results

Trial 1:

In this trial, the 1-hour incubation in 2% BSA was not implemented. Each antibody was diluted 1:500 in PBS.

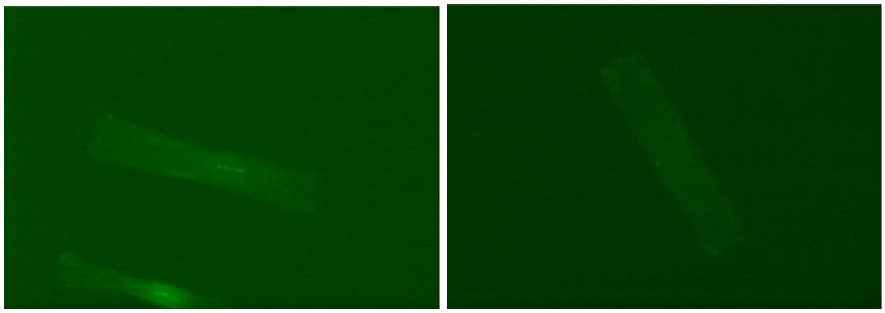

Figure 1: Images of HUASMCs using a 1:500 dilution in PBS for both antibodies.

Fluorescence appeared faint and levels of background were high using no blocking and a 1:500 dilution for each antibody, as shown in Figure 1. This procedure was developed by another group but is not sufficient for our expectations of vinculin visibility. In an effort to improve vinculin visibility, it was determined that adjusting the secondary antibody dilutions was a necessary step for improving binding to the primary antibody.

Trial 2:

This trial did not implement the 1-hour incubation in 2% BSA. The secondary antibody was added using the following dilutions in PBS: 1:50, 1:100, 1:200, 1:250.

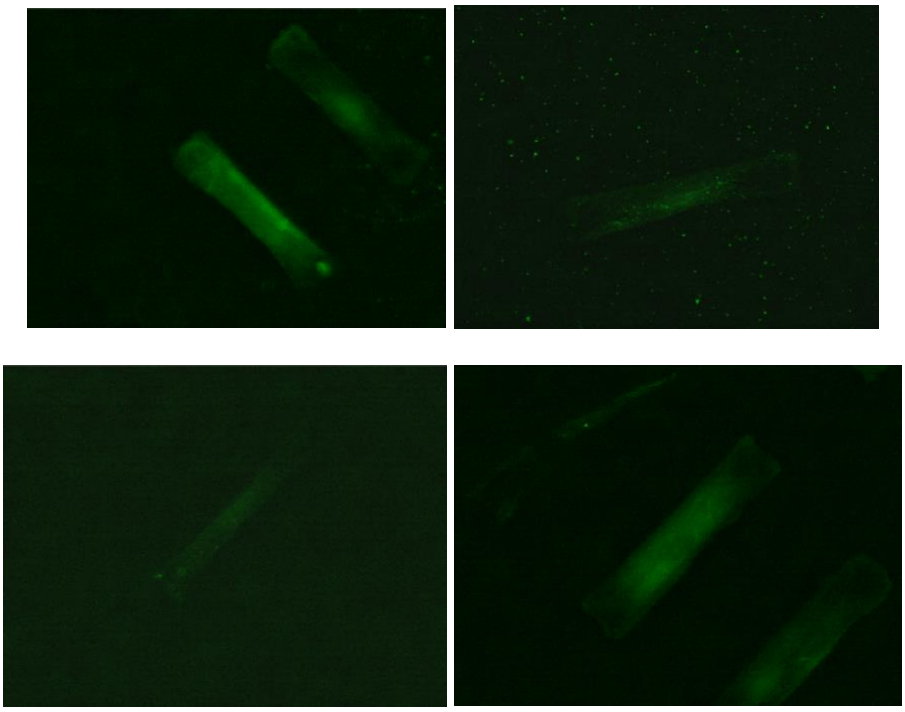

Figure 2: Image of each dilution: 1:50 (top left), 1:100 (top right), 1:200 (bottom left), and 1:250 (bottom right).

After varying the dilutions of the secondary antibody, background fluorescence was reduced but detail in the focal adhesions was still lacking (Figure 2). The relative homogeneity between dilutions tested in Figure 2 suggests that the secondary antibody concentration does not

have a significant effect on the ability to visualize the focal adhesions. This could suggest that the primary antibody did not sufficiently bind to the vinculin.

Trial 3:

This trial utilized the 1-hour incubation in 2% BSA. The primary antibody was diluted 1:100 in 2% BSA. The secondary antibody was diluted 1:200 in 2% BSA.

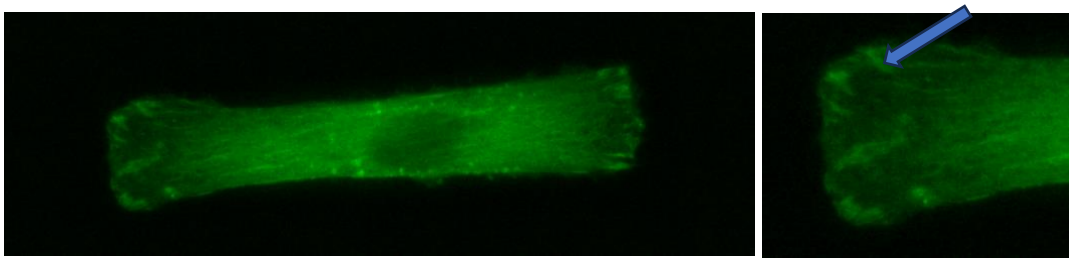

Figure 3: Image of HUASMC with primary antibody 1:100 dilution in 2% BSA. Zoomed in on focal adhesions (right). Focal adhesions are indicated with an arrow.

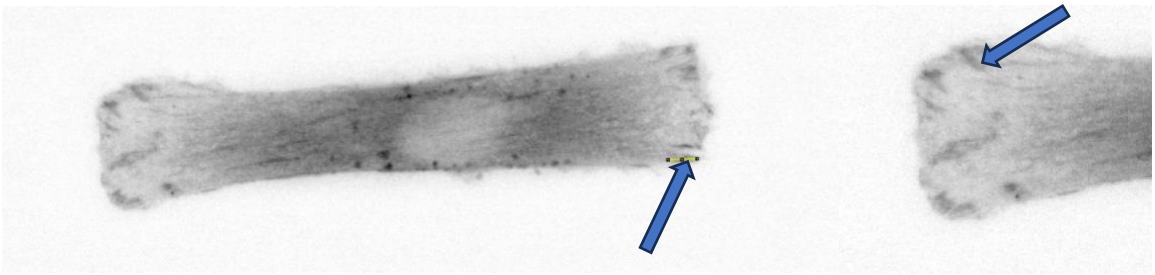

Figure 4: Inverted image used to measure focal adhesions. Focal adhesions are indicated with an arrow.

Both primary and secondary antibodies were diluted in BSA to further reduce background fluorescence. Images from trial 3, Figures 3 and 4, demonstrate that blocking using 2% BSA prevents non-specific binding, allowing for the primary antibody to bind to vinculin more efficiently. The combination of blocking, 1:100 dilution of primary antibody, and 1:200 dilution of secondary antibody was most effective for staining focal adhesions.

Table 1: Tabulation of summary statistics of focal adhesion length measured using Figure 4.

Focal Adhesion Length (μm)	
Average	32.302
Standard Deviation	11.150
Maximum	57.775
Minimum	21.095

The lengths of 8 focal adhesions, shown in Figure 4, were measured in ImageJ. The summary statistics, Table 1, demonstrate a wide range of values for focal adhesion length. Future analysis with a statistically significant sample size will provide an improved analysis of focal adhesion length and variability. However, this process optimization is an important first step in answering the question of how rate of deformation in VSMCs affects focal adhesions.

Discussion

VSMC mechanical response to deformation at various strain rates is important in understanding VSMC function changes following TBI. Recent research suggests that VSMCs do not exhibit typical viscoelastic behavior when stretched at strain rates similar to the strain rate during a TBI. Rather, stress was lower in VSMCs stretched at higher strain rates [1]. Quantification of focal adhesion size and quantity is important to understand VSMC deformation behavior because the high strain rates may cause focal adhesions to break. This is one factor that could contribute to the decreases in stress observed in VSMCs stretched at high rates. Immunofluorescence labeling of vinculin was performed to quantify focal adhesions in VSMCs. This experiment explores the effectiveness of mouse vinculin monoclonal antibody binding under various conditions. The first trial used equal concentrations of each antibody in PBS. The second

trial used a constant primary antibody concentration and a varying secondary antibody concentration, both in PBS. Trials 1 and 2 proved insufficient in staining vinculin. The findings from trials 1 and 2 suggest that the primary antibody did not effectively bind to vinculin in the VSMCs. Additionally, it was learned that secondary antibody concentration does not affect the ability to visualize focal adhesions.

The findings from the final trial indicate an improvement in primary antibody binding after an incubation period in 2% BSA. The improvement in fluorescence quality proves that BSA effectively prevents non-specific antibody binding. This allows for the primary antibody to properly bind to vinculin. Focal adhesion length was quantified for unstretched VSMCs. The average length of 8 focal adhesions was 32.302 μm. Moving forward, the staining procedure developed in this set of experiments will be applied to VSMCs strained at increasing rates. The average number of focal adhesions per cell and the average size of focal adhesions per cell for each strain rate condition will be compared to determine if the deformation rate affects focal adhesions.

Conclusion

Throughout this semester, I optimized the procedure for immunofluorescence labeling of vinculin in a research setting. Supply chain issues at times made acquiring the required reagents difficult and prevented the study of the effect of strain rate on focal adhesion size. Moving forward, this optimized procedure will be used to collect focal adhesion data on VSMCs strained at increasing rates. Overall, it was learned that immunofluorescence labeling of vinculin without preventing non-specific binding does not allow for proper binding of primary antibody to vinculin. The methods described are an important optimization of a protocol that will be used frequently

while answering the question of how VSMCs are affected by trauma. Inducing VSMCs to various deformation conditions, while staining for vinculin, may offer compelling evidence regarding the effect of focal adhesions on VSMC mechanical behavior.

References

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